AWARD NUMBER: W81XWH-16-1-0469

TITLE: Annotating MYC Status in Treatment-Resistant Metastatic Castration-Resistant Prostate Cancer With Gallium-68 Citrate PET

PRINCIPAL INVESTIGATOR: Rahul Aggarwal

RECIPIENT: University of California San Francisco
San Francisco, CA 94103

REPORT DATE: September 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
September 2017	Annual	1 Sep 2016 - 31 Aug 2017

4. TITLE AND SUBTITLE

Annotating MYC Status in Treatment-Resistant Metastatic Castration-Resistant Prostate Cancer With Gallium-68 Citrate PET

5a. CONTRACT NUMBER
W81XWH-16-1-0469

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Rahul Aggarwal (Study PI)
rahul.aggarwal@ucsf.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)

University of California San Francisco
1600 Divisadero Street, A717
San Francisco, CA 94143

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release;
Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have initiated the first-ever, proof-of-concept, translational study of gallium citrate PET as a non-invasive imaging biomarker of MYC activity for mCRPC patients receiving a next-generation potent bromodomain 4 (BRD4) inhibitor. Our preliminary results to date demonstrate that gallium citrate PET can feasibly detect metastatic lesions in patients with advanced prostate cancer, and that there are early signals that uptake on PET scan is associated with histologic evidence of small cell/neuroendocrine differentiation, MYC overexpression, and amplification of MYC gene upon analysis of circulating tumor DNA. We have observed significant intra- and inter-patient heterogeneity of gallium citrate uptake on PET scan, which is likely reflective of the underlying biological heterogeneity of metastatic castration resistant prostate cancer. We have initiated paired gallium citrate PET imaging in patients being treated with BET bromodomain inhibitors, a class of therapies known to down-regulate MYC expression. Study accrual is ongoing and interim analysis is planned within the next 6 months. Planned analyses include: 1) correlation of gallium citrate uptake on PET with MYC copy number and expression levels, and 2) determination of the mean percent change from baseline in Ga-citrate uptake on PET upon treatment with BET bromodomain inhibitor treatment.

15. SUBJECT TERMS

Prostate cancer; molecular imaging, biomarker, MYC oncogene, BET bromodomain inhibitor, castration-resistance, neuroendocrine prostate cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU		**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

TABLE OF CONTENTS

	Page No.
1. Introduction	5
2. Keywords	5
3. Accomplishments	6
4. Impact	10
5. Changes/Problems	12
6. Products	14
7. Participants & Other Collaborating Organizations	16
8. Special Reporting Requirements	19
9. Appendices	19

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The biology of metastatic castration-resistant prostate cancer (mCRPC) has shifted towards an aggressive phenotype with frequency neuroendocrine differentiation upon the emergence of resistance to abiraterone. There is an urgent unmet medical need to develop novel targeted anti-cancer therapies to improve survival for this oft-lethal disease subtype. Our preliminary data from patient-derived abiraterone-resistant metastatic tumor samples has identified significant up-regulation of the MYC oncogenic activity as a potential driver of therapeutic resistance. Emerging MYC-targeted therapies including bromodomain inhibition to down-regulate MYC expression are entering clinical studies in abiraterone-resistant mCRPC. **Critical to the successful development of MYC targeted therapies in mCRPC is the concurrent development of a functional biomarker of MYC activity to optimize dose and patient selection in early phase clinical trials. We have initiated the first-ever, proof-of-concept, translational study of gallium citrate PET as a non-invasive imaging biomarker of MYC activity for mCRPC patients receiving a next-generation potent bromodomain 4 (BRD4) inhibitor.**

Specific Aims:
(1) To compare ^{68}Ga-citrate uptake on PET imaging with level of MYC transcriptional activity assessed in abiraterone-resistant CRPC. Gallium citrate PET will be coupled with genomic analysis of mCRPC biopsies and circulating tumor DNA and tumor cells to validate Ga-68 citrate as functional biomarker of MYC oncogenic signaling.
(2) To perform first-ever proof-of-concept studies investigating the utility of Gallium citrate PET as a pharmacodynamic and predictive biomarker of MYC pathway inhibition in mCRPC. Correlative pre- and post-treatment Gallium citrate PET imaging will be integrated into an upcoming clinical trial at UCSF aimed at down-regulating MYC expression in mCRPC via BET inhibition (GS-5829).

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Prostate cancer; molecular imaging, biomarker, MYC oncogene, BET bromodomain inhibitor, castration-resistance, neuroendocrine prostate cancer

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Task 1: Finalize Imaging Protocol and Preparation for Study Opening	Months	Status
Optimize time and dose of gallium citrate in mCRPC patients under existing pilot imaging study protocol	1-6	Completed
Quality assurance of gallium generator and verification of organ dosimetry	1-6	Completed
Finalize clinical trial agreements (CTAs) with Gilead Sciences	1-4	Completed
Draft and finalize imaging protocol and imaging manual	6-9	Completed
Draft and finalize laboratory manual including collection and processing of blood and tissue under auspices of WCDT protocol	1-6	Completed
Obtain UCSF IRB approval for companion imaging study of gallium citrate PET	6-9	Completed
Obtain Investigational New Drug (IND) application	6-9	Completed
Preparation of electronic case report forms within encrypted Oncore® database	6-9	Completed
Milestone Achieved: Finalization of study contract and budget	9	Completed
Milestone Achieved: Research staff trained	9	Completed
Milestone Achieved: First patient consented, screened, and enrolled on study	9	Completed

Major Task 2: Patient Accrual/Data Monitoring (N = 20 patients over months 9-28)	Months	Status
Weekly review of active study patients for adverse events/safety monitoring (N = 20 patients)	9-28	Ongoing
Clinical data entry into encrypted Oncore® database	9-28	Ongoing
Genomic analysis of tumor biopsies in collaboration with UC Santa Cruz	9-28	Ongoing
Gallium-68 citrate PET image processing and calculation of $SUV_{max\text{-}ave}$ across metastatic lesions	9-28	Ongoing
Monthly teleconference with Study Investigators	9-28	Ongoing
Data monitoring and auditing by independent Data Monitoring and Safety Committee	9-28	Ongoing
Interim safety and efficacy data analysis	15-18	Ongoing
Milestone Achieved: Interim safety and efficacy analysis completed	18	Partially completed
Monitor patient accrual and amend protocol to adjust eligibility criteria and study procedures as needed	18-28	Ongoing
Milestone Achieved: Last patient enrolled	28	Not yet completed

Major Task 3: Study Follow-Up and Data Analysis	Months	Status
Weekly safety review of patients continuing to receive protocol therapy	28-36	Not yet completed
Patient follow up until time of completion of follow up scan	28-36	Not yet completed
Milestone Achieved: Last patient completes study follow up scan	36	Not yet completed
Assess correlation between SUVmax on gallium citrate PET with tissue-based genomic markers of MYC pathway activation in conjunction with UC Santa Cruz bioinformatics group	32-36	Ongoing
Assess percent change from baseline on gallium citrate PET upon treatment with bromodomain inhibitor	32-36	Ongoing
Milestone Achieved: Complete statistical analysis of results	34	Not yet completed
Milestone Achieved: Publication of study results in peer reviewed medical journal	36	Partially completed

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We have completed pilot imaging study to optimize the time and dose of gallium citrate in mCRPC patients. We obtained a Investigational New Drug application and Institutional Review Board approval for the study of gallium citrate PET in prostate cancer patients. We have determined the optimal time point to maximize tumor-to-background uptake at 4 hours post-injection. The optimal dose of 68-gallium citrate is 10 mCi. Patients have been been scanned in both fed and fasting state, without a difference in tumor uptake. We have verified organ dosimetry and found it to be consistent with reported findings of gallium citrate PET in other non-cancer related clinical studies. We published these pilot imaging data with gallium citrate PET imaging in mCRPC (Behr et al. Molecular Imaging and Biology 2016).

Following completion of the pilot imaging study, we have obtained IRB approval to initiate the proposed imaging studies outlined in Major Task 2 above, with coupled metastatic tumor biopsies obtained under the auspices of the WCDT/PCF/SU2C-funded project (PI: Small). To date, we have obtained tumor biopsies and circulating tumor DNA collection from 11 out of 20 planned patients with prior resistance to abiraterone. Patients underwent gallium citrate PET imaging prior to biopsy. Thus far, we have observed preliminary evidence of correlation between MYC overexpression and inferrred transcriptional activity, along wth amplification of MYC in analysis of tumor DNA, in gallium citrate PET-avid lesions. Patients with hyperamplified MYC on analysis of circulating tumor DNA had a higher percentage of PET-avid metastatic lesions (68% vs. 47%, $p < 0.05$). We have observed marked inter- and intra-patient heterogeneity with

respect to Ga-citrate uptake, which may be indicative of underlying biologic heterogeneity. Notably, we have detected evidence of neuroendocrine differentiation in several metastatic biopsies, coupled with marked PET avidity on paired gallium citrate PET scan. We published these preliminary data linking gallium citrate PET avidity in patient scans with evidence of MYC hyperactivity earlier this year (Aggarwal et al. Molecular Cancer Res 2017).

With respect to completion of Specific Aim 2, we have obtained IRB approval to perform gallium citrate PET scans before and folllwing initiation of treatment with the BET bromodomain inhibitor in patients with metastatic castration resistant prostate cancer. Thus far, we have successfully completed paired pre/post gallium citrate PET scans in 5 patients who have undergone treatment with BET bromodomain inhibitor. We have observed preliminary evidence of down-regulation of tracer uptake with the application of BET inhibitor treatment, however results are still preliminary.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

We have presented our initial findings to the Prostate Cancer Foundation Annual Scientific Meeting as well as Prostate Cancer Foundation Journal Club. We have additionally submitted UCSF press release announcing the publication of our pilot imaging study.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We aim to continue to accrue patients to the clinical imaging study, with the goal of achieiving a total of 20 evaluable patients as per the planned study design. Following completion of accrual, we will formally analyze the data in aggregate. Planned analyses include: 1) correlation tests between PET avidity with MYC amplification in cell free DNA and overexpression in metastatic tumor biopsies, 2) determination of change from baseline in tracer uptake upon treatment with BET bromodomain inhibitor treatment.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The encouraging preliminary results demonstrating the feasiblity of gallium citrate PET imaging have spurred the incorporation of this imaging modality into other planned and ongoing clinical trials in metastatic prostate cancer patients, including upcoming study of the CDK 4/6 inhibitor ribociclib in combination with docetaxel (NCT02494921). We have also integrating paired imaging with gallium citrate PET into another Phase 1 clinical trial of an alternative BET bromodomain inhibitor, ZEN-003694, which has favorable pharmacokinetic properties.

In addition, the imaging findings reported above have provided strong suppot for the use of Transferrin-based PET imaging in prostate cancer, and have spurred the clinical development of a second radiotracer, Zirconium-89 Transferrin, as an additional molecular imaging probe with the potential for a high degree of specificity and sensiivity in the detection of MYC-hyperactive mCRPC tumors. The planned first-in-human phase 1 pilot imaging study with this compound is scheduled to open to patient accrual in Q4 2017.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The encouraging findings reported above have spurred the development of gallium citrate PET imaging in other malignancies with evidence of MYC hyperactivity, including glioblastoma and hepatocellular carcinoma. The imaging group has published the first-ever pilot imaging study of gallium citrate PET in HCC patients [manuscript under review].

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*

- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  1. Aggarwal R, Behr S, Paris P, Truillet C Parker M, Huynh LT, Wei J, Hann B, Youngren J, Premasekharan G, Huang J, Ranatunga N, Chang E, Gao KT, Ryan CJ, Small EJ, and Evans MJ. Real time transferrin-based PET detects MYC-positive prostate cancer. Molecular Cancer Research 2017 [published online 7 June 2017].
  Acknowledgment of federal support: yes

  2. Behr S, Aggarwal R, Seo Y, Aparici CM, Chang E, Gao KT, Tao DH, Small EJ, and Evans MJ. A feasibility study showing 68Ga-Citrate PET detects prostate cancer. Molecular Imaging and Biology 2016;18(6):946-51.
  Acknowledgment of federal support: yes

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report.

  Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year*

(international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

1. Prostate Cancer Foundation Annual Scientific Meeting 2016, Carlsbad, CA

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

The imaging methodology for gallium citrate PET imaging was published in our pilot imaging study as described above.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

The metastatic tumor biopsies and circulating tumor DNA that are being collected under the auspices of this grant will be broadly available for additional research inquiries, along with the clinical annotation of patient outcomes.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

1) Rahul Aggarwal
Project Role: Principal Investigator
Researcher Identifier (eRA Commons ID): RAHULA
Nearest person month worked: 1
Contribution to project: Dr. Aggarwal has served as Principal Investigator of the clinical imaging study of gallium citrate PET in mCRPC patients. He has been primarily responsible for patient accrual, data analysis, and coordination for acquisition of tissue/blood samples for the correlative studies. He has contributed to data analysis and manuscript preparation.

2) Spencer Behr
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to project: Dr. Behr is the principal nuclear medicine radiologist on the project. He has been responsible for the image analysis for summary of PET scan findings. He has been involved with data analysis and manuscript preparation.

3) Michael Evans
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to project: Dr. Evans is the chief laboratory investigator involved with the project. He has contributed to the data analysis and interpretation, along with preparation of manuscript.

4) Eric Small
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to project: Dr. Small has been the principal investigator for the biopsy acquisition protocol and has overseen efforts to obtain tissue and blood collection for the correlative assays for this proposal.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

APPENDIX 1

Real time transferrin-based PET detects

MYC-positive prostate cancer

Rahul Aggarwal[1,3]*, Spencer C. Behr[2]*, Pamela Paris[3], Charles Truillet[2], Matthew F.L. Parker[2], Loc T. Huynh[2], Junnian Wei[2], Byron Hann[3], Jack Youngren[1], Jiaoti Huang[4], Nimna Ranatunga[3], Emily Chang[1], Kenneth T. Gao[2], Charles J. Ryan[1,3], Eric J. Small[1,3], and Michael J. Evans[2,3,5]

* These authors contributed equally
1. Department of Medicine, Division of Hematology/Oncology, University of California San Francisco, San Francisco, CA
2. Department of Radiology, University of California San Francisco, San Francisco, CA
3. UCSF Helen Diller Family Comprehensive Cancer Center, San Francisco, CA
4. Department of Pathology, Duke University, Durham, North Carolina
5. Department of Pharmaceutical Chemistry, University of California San Francisco, CA

Corresponding Author:

Michael J. Evans, PhD
185 Berry Street
San Francisco, CA 94107
Phone: (415) 353-3442
Email: Michael.Evans@ucsf.edu

Edited running title: PET Imaging of MYC-positive Tumors

Keywords: MYC, oncogene, PET, molecular imaging, transferrin receptor, gallium

Financial Support: This research was supported by a Stand Up To Cancer - Prostate Cancer Foundation Prostate Dream Team Translational Research Grant (SU2C-AACR-DT0812, PI: E.J.S.). This research grant is made possible by the generous support of the Movember Foundation. Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research. M.J.E. and R.A are Young Investigator Awardees from the Prostate Cancer Foundation. M.J.E. was supported by the National Institutes of Health (R00CA172695, R01CA17661), a Department of Defense Idea Development Award (PC140107), the UCSF Academic Senate, and GE Healthcare. C.T. was supported by a postdoctoral fellowship from the Department of Defense Prostate Cancer Research Program (PC151060). M.F.L.P. was supported by a postdoctoral fellowship from the Department of Defense Prostate Cancer Research Program (PC161005). Research from UCSF reported in this publication was supported in part by the National Cancer Institute of the National Institutes of Health under Award Number P30CA082103. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ABSTRACT

Non-invasive biomarkers that detect the activity of important oncogenic drivers could significantly improve cancer diagnosis and management of treatment. The goal of this study was to determine if ^{68}Ga-citrate (which avidly binds to circulating transferrin) labeled transferrin (Tf) can detect MYC-positive prostate cancer tumors, since the transferrin receptor is a direct MYC target gene. Positron emission tomography (PET) imaging paired with ^{68}Ga-citrate and molecular analysis of preclinical models, human cell-free DNA (cfDNA) and clinical biopsies were conducted to determine whether ^{68}Ga-citrate can detect MYC-positive prostate cancer. Importantly, ^{68}Ga-citrate detected human prostate cancer models in a MYC-dependent fashion. In patients with castration resistant prostate cancer (CRPC), analysis of cfDNA revealed that all patients with ^{68}Ga-citrate avid tumors had a gain of at least one MYC copy number. Moreover, biopsy of two PET avid metastases showed molecular or histological features characteristic of MYC hyperactivity. These data demonstrate that ^{68}Ga-citrate targets prostate cancer tumors with MYC hyperactivity. A larger prospective study is ongoing to demonstrate the specificity of ^{68}Ga-citrate for tumors with hyperactive MYC.

Implications: Non-invasive measurement of MYC activity with quantitative imaging modalities could substantially increase our understanding of the role of MYC signaling in clinical settings for which invasive techniques are challenging to implement or do not characterize the biology of all tumors in a patient. Moreover, measuring MYC activity

non-invasively opens the opportunity to study changes in MYC signaling in patients under targeted therapeutic conditions thought to indirectly inhibit MYC.

INTRODUCTION

The MYC oncogene is an important mediator of tumor initiation and progression in prostate cancer(1-3). In patients with metastatic castration resistant prostate cancer (mCRPC), recent data implicate MYC (and closely related oncogene MYCN) in the emergence of treatment-associated small cell/neuroendocrine prostate cancer (tSCNC). t-SCNC is a lethal disease subset that is increasing in prevalence in response to androgen ablasting therapy (4, 5). Preclinical studies with prostate cancer models demonstrating that MYC hyperactivation in concert with PI3K/Akt/mTOR signaling can drive neuroendocrine differentiation and visceral metastasis formation provide support for a potential causal role of MYC in promoting tumor progression to an aggressive mCRPC phenotype(6).

MYC was previously thought to be 'undruggable'; however, multiple classes of therapies are now aimed at indirectly inhibiting MYC or its downstream mediators. Foremost among them are bromodomain extra terminal (BET) inhibitors, which have demonstrated the ability to directly downregulate MYC expression. These inhibitors also display significant antitumor activity in a variety of androgen-independent, MYC-high prostate cancer cell lines and xenograft models(7-9). Multiple early-phase clinical trials of BET inhibitors in abiraterone/enzalutamide-resistant mCRPC are underway (e.g. NCT02705469, NCT02607228). Additional drugs targeting effectors of MYC signaling, including cyclin-dependent kinase (CDK) and PIM kinases, are also in pre-clinical and clinical development in prostate cancer and other solid tumor malignancies(10, 11).

Similar to how ^{18}F-dihydrotestosterone (DHT) PET fostered the development of the androgen receptor (AR) antagonists enzalutamide and apalutamide(12, 13), the

burgeoning number of therapies aimed at inhibiting MYC underscores the need to develop a companion imaging biomarker capable of monitoring MYC transcriptional activity in real-time, both to identify treatment naïve patients whose tumors harbor hyperactive MYC, and to enable longitudinal assessment of MYC pathway modulation and therapeutic response.

MYC transcribes the transferrin receptor (TFRC)(14). TFRC-targeting radiolabeled analogues may therefore serve as a non-invasive quantitative measurement of MYC transcriptional activity. Consistent with this hypothesis, we have previously shown that ^{89}Zr coupled to transferrin via the chelator desferrioxamine is retained in cancer models in a MYC dependent fashion(15-18). The goal of this study was to determine if ^{68}Ga-citrate, a human ready radiotracer that measures TFRC expression levels in vivo(19), could be used to detect MYC positive prostate cancer models and metastases (^{89}Zr-transferrin is not yet cleared for human use). . Building on a pilot clinical study in which we showed that ^{68}Ga-citrate is taken up in human prostate cancer metastases(20), we conducted the first preclinical tumor imaging studies with ^{68}Ga-citrate, as well as the first patient studies of ^{68}Ga-citrate PET imaging coupled with analysis of cell free DNA (cfDNA) and paired metastatic tumor biopsies to investigate whether ^{68}Ga-citrate can detect CRPC with MYC hyperactivity and neuroendocrine differentiation.

MATERIALS AND METHODS

General methods:

PC3 and 22Rv1 cells were obtained from ATCC and subcultured according to manufacturer's recommendations. (+)-JQ1 was a generous gift from Dr. James Bradner. iBET-151 was purchased from Selleckchem and used without further purification. Human holo-transferrin was purchased from Sigma Aldrich and succinimidyl-DFO was obtained from Macrocyclics (Dallas, TX). Zirconium-89 was purchased from 3D Imaging, LLC (Maumelle, AR). ^{68}Ga-citrate was produced by the cyclotron core at UCSF and administered in buffered PBS. Antibodies against TFRC (Santa Cruz Biotechnology), MYC (Abcam), and Actin (Sigma) were utilized for Western blot analyses.

Radioiodination of transferrin and in vitro uptake assays:

Iodination with iodine-125 was done in pre-coated iodination tubes (Pierce). 100 mg of transferrin was dispersed in 200 μL of PBS solution and added to the pre-coated iodination tubes. In a separate eppendorf, 1 μL of HCl (0.2 M), 2.5 μL of phosphate buffer (0.5 M, pH = 8), 10 μL of potassium iodide solution (1 mg/ml) was prepared. 3 mCi of iodine-125 (Perkin Elmer) was added into the tubes and the previous solution was then mixed in the iodination tubes. After 15 min of reaction at room temperature the solution was purified via PD10 column pre equilibrated with 20 mL of PBS solution. The purity was assessed *via* iTLC, and ^{125}I-Tf was always > 98% pure.

PC3 and 22Rv1 cells were counted and plated at fixed cell concentrations between treatment arms. Cells were treated with vehicle or the bromodomain inhibitors iBET-151 or JQ1 (1 μM) for 48 hours, whereupon they were washed and incubated with 10 μCi ^{125}I-Tf for 30 min at 37^{o} C. After washing twice with PBS, the cell associated

activity was harvested in 1M NaOH (aq.). The cell associated activity was expressed as a % of total activity to which the cells were exposed. This value was further normalized to cell number to correct for treatment-induced changes in cell viability.

In vitro studies of MYC expression:

PC3 and 22Rv1 cells (4 x 10^5) were incubated with vehicle, (+)-JQ-1 (1 μM) and IBET-151 (1 μM) at 37 degrees Celsius for 48 hours. Cells were lysed, and the mRNA was extracted using the Quick-StartProtocol of the Qiagen. The mRNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR analysis was performed using PikoReal 96 Real-Time PCR System. MYC and GADPH expression (serving as housekeeper gene) levels were probed using validated seqeunces, and the relative ratio of the expression of the two genes was measured.

Animal studies:

All animal studies were conducted in compliance with the Institutional Animal Care and Use Committee at UCSF. ^{89}Zr-Tf was prepared as previously reported(16). Three to five week old male *nu/nu* mice and C57BL/6 mice were obtained from Charles River. Nu/nu mice were inoculated with 1 x 10^7 22Rv1 or PC3 cells subcutaneously into one flank in a 1:1 mixture (*v/v*) of media and Matrigel (Corning). Tumors were palpable within 14-21 days after injection. The drugs were suspended in HPMT solution (0.5% *w/v* hydroxypropyl-methylcellulose dissolved in water plus 0.2% *v/v* Tween 80). Tumor bearing mice were treated once daily via oral gavage with iBET-151 (30 mg/kg/d), (+)-

JQ1 (50 mg/kg, BID), and vehicle for 3 days prior to radiotracer injection. Drug treatment continued during the 48 hours that ^{89}Zr-Tf distributed in the body. C57BL/6 mice were treated with 50 μL of neat turpentine in the right hindlimb and injected 24 hours later with ^{68}Ga-citrate.

Small-animal PET and biodistribution studies:

Tumor bearing mice (n=5 per treatment arm) received ~400 μCi of ^{89}Zr-Tf or ^{68}Ga-citrate in 100 μL volume intravenously using a custom mouse tail vein catheter with a 28-gauge needle and a 100-150 mm long polyethylene microtubing (0.28 mm I.D. × 0.64 mm O.D., Scientific Commodities, Inc., Lake Havasu City, AZ). Approximately 32 μg of ^{89}Zr-Tf at a specific activity of 0.4 mCi/nmol was administered per mouse. The mice were imaged on a dedicated small animal PET/CT scanner (Inveon, Siemens Healthcare, Malvern, PA). Mice were imaged at 4 or 48 hours post injection. Animals were scanned for 20-40 minutes for PET, and the CT acquisition was 10 minutes. The coregistration between PET and CT images was obtained using the rigid transformation matrix from the manufacturer-provided scanner calibration procedure since the geometry between PET and CT remained constant for each of PET/CT scans using the combined PET/CT scanner. Animals were anesthetized with gas isoflurane at 2% concentration mixed with medical grade oxygen. PET data were framed dynamically for the first two time points. The durations of the 0.5-h PET data were: 10×10s, 5×40s, 1×300s, and 5×600s. The 3-5 h PET data were also divided to two 1800s frames. The in vivo CT parameters were 120 projections of continuous rotations to cover 220° with an x-ray tube operated at 80 kVp, 0.5 mA, and 175 ms exposure time.

Manufacturer-provided ordered subsets expectation maximization (OS-EM) algorithm was used for PET reconstruction that resulted in 128×128×159 matrices with a voxel size of 0.776×0.776×0.796 mm^3. The CT image was created using a conebeam Feldkamp reconstruction algorithm (COBRA) provided by Exxim Computing Corporation (Pleasanton, CA). The matrix size of the reconstructed CT images was 512×512×662 with an isotropic voxel size of 0.191×0.191×0.191 mm^3. The photon attenuation correction was performed for PET reconstruction using the coregistered CT-based attenuation map to ensure the quantitative accuracy of the reconstructed PET data.

To evaluate the uptake of ^{68}Ga and ^{89}Zr-Tf radiotracers in human xenografts, biodistribution studies were conducted following imaging. Animals were euthanized by CO_2 asphyxiation after scans were completed. Sixteen tissues, including the tumor, were harvested immediately following sacrifice. The tissues were weighed and counted using a Wizard3 gamma counter (Perkin Elmer) to assess ^{68}Ga or ^{89}Zr concentration. Calibration with known amounts of ^{68}Ga and ^{89}Zr was performed to determine the amount of activity in each organ. This activity was then decay corrected and the percentage of the injected dose per gram (%ID/g) of tissue was calculated and reported.

Patient selection and clinical study design:

The clinical study was approved by the UCSF institutional review board, and all patients provided written informed consent before study enrollment. All patients had histologically confirmed prostate cancer, and had developed progressive metastatic castration resistant disease by PCWG2 criteria. All had progressive disease following treatment with abiraterone and/or enzalutamide(21). Patients were required to undergo

conventional scans including cross-sectional imaging of the chest/abdomen/pelvis and whole body radionuclide bone scan (either ^{99m}Tc- HDP or ^{18}F-NaF) within 12 weeks prior to ^{68}Ga-citrate PET imaging. All included patients had imaging evidence of at least three metastatic lesions by conventional imaging. On the day of ^{68}Ga-citrate PET imaging, prior to tracer injection, patients had peripheral blood collected for determination of cell free tumor DNA MYC copy number status. A subset of patients underwent CT-guided core needle biopsy of an accessible metastatic lesion performed within 4 weeks following completion of ^{68}Ga-citrate PET imaging. Patients underwent an optional second ^{68}Ga-transferrin PET scan within 12 weeks of firstscan.

PET imaging protocol

Patients were injected with up to 15 mCi (555 MBq) (average 7.42 mCi [274.6 MBq], range 3.7 to 11.9 mCi [136.9 to 438.5 MBq]) ^{68}Ga-citrate intravenously. PET acquisition was acquired between 120 and 263 minutes after injection (average 210 minutes). Images were acquired on either a PET/CT or PET/MR. PET/CT examinations were performed on either a Biograph 16 (Hi-Rez) PET/CT scanner (Siemens AG, Erlangen, Germany) with an integrated PET and 16-MDCT scanner or a Discovery VCT PET/CT scanner (GE Medical Systems, Milwaukee, WI) with an integrated PET and 64-MDCT scanner. A low-dose CT was acquired for PET attenuation correction. PET/MR images were performed on a SIGNA PET/MR (GE Medical Systems, Milwaukee, WI). Attenuation correction for PET reconstruction was performed using a MR-based attenuation correction (MRAC) technique provided by the scanner manufacturer.

Image analysis

Maximum intensity projection (MIP), axial, coronal and sagittal reconstructions and PET/MR fused images were reviewed on an Advantage Windows Workstation (AW, Waukesha, WI). PET images were evaluated by trained nuclear medicine physician blinded to the results of conventional imaging scans as well as clinical/genomic features of the case and scored for the presence of PET avid lesions. Lesions were considered PET positive if uptake was focal, greater than the adjacent background soft tissue and not in an expected physiologic structure such as the urinary bladder, vessels or salivary glands,

For semi-quantitative analysis, a volume of interest (VOI) was manually drawn around PET-avid lesions and SUV_{max} were recorded. The location of abnormal radiotracer uptake was compared to CT and nuclear medicine bone-scans. Additionally, SUV_{mean} values were recorded in the liver, paraspinous soft tissues, bone (right sacrum), and mediastinal blood pool for determination of normal structures.

With conventional imaging, soft tissue metastases were considered positive if greater than 1 cm in long axis, except for lymph nodes that were considered positive if greater than 1.5 cm in short axis. Bone lesions on radionuclide scan were considered positive if uptake was focal and not in a pattern consistent with arthritis or antecedent trauma/fracture.

Cell free DNA analysis of MYC amplification

Blood (20 ml) was collected from mCRPC patients in purple top EDTA tubes and was fixed using 10% neutral buffered solution containing 4% paraformaldehyde within 2 hrs

of receiving the sample. Plasma was isolated immediately from the fixed blood by centrifuging the samples for 10 min at 200 x g at room temperature followed by a second spin at 1600 x g for 10 min. Avoiding the buffy coat, plasma was carefully transferred to new 15 ml tubes and centrifuged at 1600 x g for an additional 10 min. The plasma was transferred to 50 ml tubes and stored at -80°C. cfDNA was extracted from plasma using the QiAamp Circulating Nucleic Acids kit (Qiagen) according to the manufacturer's protocol. The extraction was carried out within a week of the plasma extraction. The extracted cfDNA was eluted in 20 µl of AE (Qiagen) buffer and assayed for MYC copy number using Digital PCR (QuantStudio, Life Technologies). 1X QuantStudio 3D Digital PCR Master Mix V2 (Applied Biosystems), TaqMan Copy Number Assay Hs02045885_cn (MYC) and 1X RNAseP TaqMan copy number reference assay (Applied Biosystems) were used in the reaction. DNA extracted from PC3 cell lines and PC3 DNA spiked into healthy donor blood was used as positive (MYC amplified) controls while male normal germline DNA was used as negative control (MYC wild type). 6µl of cfDNA was combined with the PCR reaction mix and loaded onto a QuantStudio 3D Digital PCR 20K chip. The QuantStudio 3D Analysis Suite Software was used to evaluate MYC copy number for each patient sample based on the reference gene (RNAseP) copy number (i.e. 2 copies).

Analysis of Histologic Evidence of Small Cell Neuroendocrine Carcinoma in Metastatic Tumor Biopsies

FFPE tissue was histologically evaluated in a central CLIA-certified lab for evidence of neuroendocrine differentiation by morphologic and immunohistochemical criteria(22).

Statistical Analysis:

The percentage of injected activity taken up in prostate cancer tumors versus other organs in mice injected with either ^{68}Ga-citrate or ^{89}Zr-Tf analyzed using the unpaired, two-tailed Student's *t*-test. Differences at the 99% confidence level ($P < 0.01$) were considered to be statistically significant. The imaging data from patient studies were summarized using descriptive statistics. Fisher's exact test was used to compare frequency of PET-avid lesions across organ site.

RESULTS

^{68}Ga-citrate shows MYC dependent uptake in human prostate cancer models.

^{68}Ga-citrate has not been previously evaluated in tumor bearing mice. Therefore, we first conducted a time course study in *nu/nu* mice bearing subcutaneous PC3 xenografts, a MYC positive model of human PCa, to define the optimal time point post injection to study ^{68}Ga-citrate. Mice received 400-500 μCi of ^{68}Ga-citrate, and biodistribution studies were conducted at 2, 4, and 6 hours post injection (**Figure 1A** and **Supplemental Figure 1**). Peak tumor uptake was observed at 4 hours post injection. Moreover, blood pool activity declined from 2 – 4 hours post injection, and remained stable from 4 – 6 hours post injection. Radiotracer uptake in normal tissues (liver, spleen, kidney, and muscle) saturated within 2 hours post injection, and the level of uptake was lower than what was observed in the tumor. The highest tumor to blood and tumor to muscle ratios were found to be at 4 and 6 hours post injection (**Supplementary Table 1**). A follow-up study in mice bearing subcutaneous 22Rv1 xenografts, an androgen receptor and MYC positive

model of human prostate cancer, also showed high ^{68}Ga-citrate uptake 4 hours post injection, with similar uptake in normal mouse tissues and compartments (**Figure 1B and Supplemental Figure 2**).

Because blood pool activity was approximately equivalent with tumor uptake of ^{68}Ga-citrate in both mouse cohorts, we next tested if tumor uptake of ^{68}Ga-citrate was equivalent with that of ^{89}Zr-Tf at time points we previously showed to reflect receptor mediated binding to tumors(15-17, 23, 24). We reasoned that equivalent levels of radiotracer uptake in tumor would be strong evidence of specific receptor binding by ^{68}Ga-citrate. *Nu/nu* mice bearing subcutaneous 22Rv1 xenografts were treated with ~400 μCi of ^{89}Zr-Tf and radiotracer biodistribution was studied 48 hours post injection. Blood pool associated activity was significantly lower than that of tumor, as expected (**Figure 1C**). Moreover, tumor uptake of ^{89}Zr-Tf was equivalent to the uptake of ^{68}Ga-citrate.

To further show evidence of specific receptor binding by ^{68}Ga-citrate at 4 hours post injection, a cohort of *nu/nu* mice bearing subcutaneous PC3 or 22Rv1 tumors were pretreated with vehicle or the BET bromodomain inhibitors (+)-JQ1 or iBET-151. Both JQ1 and iBET-151 therapies down-regulated MYC mRNA and Tf uptake in vitro (**Supplemental Figure 3**). Tumors in the mice receiving 5 days of treatment had significantly less ^{68}Ga-citrate uptake (**Figure 2A** and **2B,** see **Supplemental Figures 4** and **5**). Moreover, the percent change in tumor uptake observed in BET inhibitor-treated mice was similar between ^{68}Ga-citrate and ^{89}Zr-Tf (^{89}Zr-Tf biodistribution was studied 48 hours post injection.

^{68}Ga-citrate has been used in tumor naïve humans and rodents to detect the focal accumulation of activated leukocytes due to inflammation or infection(25). On this basis,

we tested whether ^{68}Ga-citrate could quantitatively distinguish tumor from an inflammatory abscess. A cohort of tumor naïve mice were injected with ~50 μL of turpentine in the right hindlimb to induce an acute phase response, and the mice were treated with ^{68}Ga-citrate 24 hours after injection. PET and biodistribution studies conducted 4 hours post radiotracer injection showed detectable accumulation of ^{68}Ga-citrate in the inflamed muscle above the contralateral untreated hindlimb **(Figure 2C)**. The magnitude of ^{68}Ga-citrate accumulation in the inflamed muscle was statistically lower than what was observed in either prostate cancer tumor using the same imaging conditions.

Patient Imaging Results

20 patients were enrolled on study between May 2015 and December 2016. Baseline characteristics are shown in **Supplementary Table 2**. All patients had mCRPC with prior progression on abiraterone and/or enzalutamide.

A total of 326 lesions were detected on conventional imaging (CT, ^{99m}Tc-HDP), of which 53% were avid for ^{68}Ga-citrate. Roughly two thirds (63.8%) of osseous lesions were avid for radiotracer, while ~20% of lymph node and ~5% of visceral organ lesions were avid. Significant qualitative and quantitative inter-tumoral heterogeneity was observed with respect to ^{68}Ga-citrate uptake **(Figure 3)**. 174 metastatic lesions (53.3%) were PET avid and 152 (46.7%) were negative on ^{68}Ga-citrate PET imaging. The average SUV_{max} of ^{68}Ga-citrate positive lesions was 5.74 (SD 2.89, range 1.8 to 19.4). The median percentage of PET-positive lesions per patient was 50% (range 0-93%). There was no relationship between dose of ^{68}Ga-citrate or tumor uptake time with the

percentage of PET avid lesions or intra-tumoral SUV_{max}. PET avid lesions were more likely to be detected in the bone versus soft tissue (63.8% vs. 12.1%; $p < 0.0001$) **(Table 1)**. **Figure 4** highlights a particular example of a patient with heterogeneous ^{68}Ga-citrate uptake within metastatic lesions in the bony pelvis, with adjacent lesions demonstrating widely varying degree of intra-tumoral uptake.

^{68}Ga-citrate PET Uptake in Tumor and Normal Organs is Reproducible Upon Serial Imaging

Four patients underwent paired imaging with ^{68}Ga-citrate PET imaging, with a median interval of 37 days (range 30 – 39 days) between scans (**Supplementary Table 3**). During this interval, patients were clinically stable and had no significant change in serum PSA level to indicate disease progression. The average change in SUV_{mean} between patient scans for blood pool, liver, paraspinus muscles and bones was 0.38 (range = -0.4 – 0.4; SD =0.05), 0.25 (range = -0.6 to 0.1; SD = 0.24), 0.28 (range = -0.4 – 0.4; SD = 0.15), and 0.3 (range = -0.4 to 0.3; SD = 0.08). Across all PET avid metastatic lesions for these four patients (N = 10; all osseous), there was likewise no significant difference in ^{68}Ga-citrate uptake between pre- to post-scans (mean change from baseline in SUV_{max} = -0.29 (range -5.3 – 1.4; SD = 1.89).

Pronounced ^{68}Ga-citrate PET uptake is observed in a subset of mCRPC patients with high tumor MYC amplification

Eighteen of twenty patients (90%) were evaluable for MYC copy number gain by cell free DNA (cfDNA) analysis. We observed a gain of at least one copy of MYC in 16 out

of 18 (89%) evaluable samples **(Table 2)**. This is generally consistent with prior reports demonstrating high prevalence of 8q chromosome gain in prostate cancer (26, 27). All 16 patients with evidence of MYC copy gain on cfDNA analysis had at least one ^{68}Ga-citrate-avid metastatic lesion. Higher level focal MYC amplification (> 2 copy number gain) was observed in six patients (33%), also consistent with prior molecular analyses of mCRPC tumors. Though the limited sample size precludes definitive assessment of an association between MYC amplification in cfDNA with ^{68}Ga-citrate PET, the subset of 6 patients with high level MYC amplification on cfDNA demonstrated a higher percentage of ^{68}Ga-citrate-avid lesions compared to those without MYC amplification (68.8% vs. 46.6%). **Figure 5** illustrates one such patient (Patient-004) with MYC amplification on cfDNA analysis (estimated 6 copies of MYC gained) with striking PET avidity on ^{68}Ga-citrate PET, with 29 out of 32 total osseous metastases (90%) positive for uptake (average SUV_{max} = 6.3/lesion).

^{68}Ga-citrate PET detects tumors with histologic and serologic evidence of neuroendocrine differentiation

11 patients (55%) underwent CT-guided metastatic tumor biopsy following ^{68}Ga-citrate PET scan. The median time interval between PET scan and subsequent image-guided tumor biopsy was 6 days (range 4 – 38). Of the 7 patients with metastatic tissue evaluable for histologic analysis, one demonstrated pure small cell neuroendocrine differentiation on pathology review (patient-011), and the other six tumors demonstrated adenocarcinoma differentiation (Supplementary Table 4). The tumor with pure SCNC histology was located in the R femur of this patient **(Figure 6)**. On coupled ^{68}Ga-citrate

PET imaging, this lesion demonstrated high avidity for the tracer, above the median for the patient cohort (SUV_{max} = 6.9), and significantly higher than adjacent metastastic lesions **(Figure 6)**. Genomic interrogation of the metastatic tumor biopsy revealed high-level MYC amplification (estimated 4 copy number gain) consistent with prior reports of treatment-emergent small cell neuroendocrine prostate cancer. An additional patient (patient-004) had serologic evidence of neuroendocrine differentiation (serum chromogranin = 66 ng/mL; upper limit of normal = 15 ng/mL) with a concordant markedly positive PET scan with 29 out of 32 metastatic lesions positive for uptake, as previously described above **(Figure 5)**.

In the two patients with genomically evaluable paired cfDNA and metastatic tumor biopsies, it is worth noting that patient-011 demonstrating only 1 copy gain of MYC in analysis of cfDNA vs. 4 copies gained in metastatic biopsy. Patient-003, in contrast, demonstrated 3 copies of MYC gained in both metastatic biopsy and cfDNA. These results support the significant interlesional and interpatient heterogeneity observed on PET imaging and highlight the difference between lesion-specific genomic assessment and aggregated results on cfDNA analysis.

^{68}Ga-citrate uptake is recapitulated in patient-derived xenograft of a liver metastasis

We isolated tumor tissue from the liver metastasis (patient-003) and propagated it in a NOD SCID mouse model (**Supplemental Figure 7**). The patient-derived xenograft (PDX) tumor was histologically similar to the tumor biopsy **(Supplementary Figure 8)**, and demonstrates similar mRNA transcript levels (log-scale) of MYC (liver metastasis = 10.9; PDX = 10.6) and TFRC (liver metastasis = 13.2; PDX = 12.3). Upon imaging of the

PDX tumor with ^{68}Ga-citrate PET, we observed comparable intra-tumoral uptake as that observed in the liver metastasis from the patient PET scan (% ID/g = 2.3 ± 0.9 in PDX tumor; SUV_{mean} = 4.9 in liver metastasis, see **Supplemental Figure 9**).

DISCUSSION

In this report, we show that both human prostate cancer models and clinical disease bearing the molecular and/or histological features of MYC hyperactivity are detectable with ^{68}Ga-citrate PET. Our preclinical experiments show specific tumor binding of ^{68}Ga-citrate in MYC-high prostate cancer cell lines and xenograft models, and suggest a novel tool to permit functional read-out of pharmacologic inhibition of the MYC signaling pathway by BET inhibitors and perhaps other emerging therapies. In patients with mCRPC, we show that a subset of patients harbored markedly ^{68}Ga-citrate-avid tumors with concordant molecular evidence of MYC activation detectable in paired analysis of cfDNA. With biopsy, two examples are shown of patients with histologic or serologic evidence of SCNC, and they also have tumors markedly avid for ^{68}Ga-citrate. These results provide robust justification to further investigate the clinical application of ^{68}Ga-citrate PET as a real-time, noninvasive monitoring tool that can potentially capture MYC-driven prostate cancer, including tumors with SCNC histology.

MYC transcriptional activity is associated with SCNC, a subset of advanced prostate cancer that is highly lethal, and that can be difficult to identify without invasive and sometimes technically challenging metastatic biopsies. Although MYC inhibitors are under development, pharmacodynamic markers of MYC inhibition are not readily available. Therefore, these data are timely and significant, as the successful outcome of

preclinical and/or clinical trials with indirect MYC inhibitors will likely depend on biomarkers like ^{68}Ga-citrate that can identify treatment naïve tumors with MYC hyperactivity (which therefore may be most likely to respond to an anti-MYC inhibitor), as well as monitor the down-regulation of MYC activity longitudinally that might predict a clinical response to treatment.

Our pre-clinical experiments indicate a high degree of tumor specificity of ^{68}Ga-citrate for MYC-high prostate cancer cell lines and xenograft models, and suggest a novel tool to permit functional read-out of pharmacologic inhibition of the MYC signaling pathway by BET inhibitors and perhaps other emerging therapies. In patients with mCRPC, we show that the subset of patients with focal MYC amplification detectable in cfDNA harbored a higher proportion of tumors with ^{68}Ga-citrate-avid avidity. We further demonstrate that ^{68}Ga-citrate PET intra-tumoral uptake is reproducible but with appreciable inter-tumoral and inter-patient heterogeneity that may reflect divergent clonal evolution with varying degree of MYC hyperactivation in the treatment-resistant mCRPC setting (28). Whole body ^{68}Ga-citrate PET imaging may therefore represent a valuable novel tool to annotate the degree of inter-tumoral heterogeneity with respect to MYC/neuroendocrine signaling in a non-invasive, real-time fashion.

The underlying reason for the preferential ^{68}Ga-citrate PET avidity in bone lesions relative to soft tissue metastases is not readily apparent. There are no significant differences in TFRC expression levels by anatomic site of disease in mCRPC in ours and other publicly available datasets. One prior report has suggested that ^{68}Ga-citrate accumulation is due to regional variation in perfusion and non-receptor mediated accumulation in regions of leaky vasculature(27). The consistency in ^{68}Ga-citrate uptake

between our PDX subcutaneous model and *in situ* liver metastasis, however, supports tumor-specific uptake as opposed to regional vascular permeability as the predominant determinant of radiotracer accumulation. Additional studies in CRPC patients harboring soft tissue metastases will be necessary to fully characterize the expected pattern of ^{68}Ga-citrate PET uptake in non-osseous sites of metastases.

Asangani et al. previously did not observe any down regulation of MYC in PC3 cells when treated with JQ1(8). Reviewing each study carefully, we hypothesize that methodological differences in the experiments may account for the apparent discrepancy with our data. Asangani et al treated PC3 cells for 24 hours with JQ1 and dosed at concentration of 500 nM, which did not reduce MYC protein levels by Western blot. In our study, we treated PC3 cells with JQ1 at a dose of 1 μM for 48 hours, which did reduce MYC mRNA. Our higher dose and longer drug exposure may account for why we observe anti-MYC effects that the previous authors did not report. Importantly, we also show anti-MYC effects in PC3 with a structurally discrete BET bromodomain inhibitor.

MYC hyperactivity in other cancers has also been associated with specific imaging patterns. Palaskas et al. reported that ^{18}F-FDG specifically demarcated basal like breast cancer with MYC hyperactivity(28). It is unclear if there is a relationship between MYC and ^{18}F-FDG avidity in prostate cancer, particularly given that CRPC is not avid for ^{18}F-FDG in most circumstances.

The case examples discussed demonstrate the potential utility of ^{68}Ga-citrate PET as a detection and treatment response monitoring tool for therapies applied against the MYC signaling pathway in SCNC. Given the likely growing prevalence of SCNC as a

resistance mechanism to potent androgen signaling blockade, and the lack of validated methods to identify SCNC differentiation other than metastatic tumor biopsy, there is a clear need to develop non-invasive biomarkers to detect and monitor treatment response in this highly aggressive, lethal disease subset. Our studies with ^{68}Ga-citrate come at an opportune time given the recent FDA approval of ^{68}Ga-DOTA-TATE for the detection of neuroendocrine tumors. Preliminary reports with DOTA-TATE imaging in CRPC indicate potential utility of detecting lesions with neuroendocrine differentiation(29). However the range of uptake is modest, and it is unclear whether DOTA-TATE avidity will be observed in higher grade tumors, analogous to the inverse correlation between tracer uptake and tumor grade observed with neuroendocrine tumors of GI origin. Further patient studies with paired ^{68}Ga-citrate PET scan coupled with tumor biopsies to explore the relationship between SCNC differentiation and ^{68}Ga-citrate uptake on PET scan are underway.

Acknowledgements:

This research was supported in part by a Stand Up To Cancer Dream Team award, grant number SU2C-AACR-DT0812 (PI: E.J.S.). This research grant is administered by the American Association for Cancer Research, the scientific partner of SU2C. M.J.E. and R.A are Young Investigator Awardees from the Prostate Cancer Foundation. M.J.E. was supported by the National Institutes of Health (R00CA172695, R01CA17661), a Department of Defense Idea Development Award (PC140107), the UCSF Academic Senate, and GE Healthcare. C.T. was supported by a postdoctoral fellowship from the Department of Defense Prostate Cancer Research Program

(PC151060). M.F.L.P. was supported by a postdoctoral fellowship from the Department of Defense Prostate Cancer Research Program (PC161005). Research from UCSF reported in this publication was supported in part by the National Cancer Institute of the National Institutes of Health under Award Number P30CA082103. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References:

1. Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, et al. Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer cell. 2003;4:223-38.

2. Cho H, Herzka T, Zheng W, Qi J, Wilkinson JE, Bradner JE, et al. RapidCaP, a novel GEM model for metastatic prostate cancer analysis and therapy, reveals myc as a driver of Pten-mutant metastasis. Cancer discovery. 2014;4:318-33.

3. Koh CM, Bieberich CJ, Dang CV, Nelson WG, Yegnasubramanian S, De Marzo AM. MYC and Prostate Cancer. Genes & cancer. 2010;1:617-28.

4. Beltran H, Rickman DS, Park K, Chae SS, Sboner A, MacDonald TY, et al. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. Cancer discovery. 2011;1:487-95.

5. Aparicio A, Tzelepi V. Neuroendocrine (small-cell) carcinomas: why they teach us essential lessons about prostate cancer. Oncology. 2014;28:831- 8.

6. Lee JK, Phillips JW, Smith BA, Park JW, Stoyanova T, McCaffrey EF, et al. N-Myc Drives Neuroendocrine Prostate Cancer Initiated from Human Prostate Epithelial Cells. Cancer cell. 2016;29:536-47.

7. Asangani IA, Wilder-Romans K, Dommeti VL, Krishnamurthy PM, Apel IJ, Escara-Wilke J, et al. BET Bromodomain Inhibitors Enhance Efficacy and Disrupt Resistance to AR Antagonists in the Treatment of Prostate Cancer. Molecular cancer research : MCR. 2016;14:324-31.

8. Asangani IA, Dommeti VL, Wang X, Malik R, Cieslik M, Yang R, et al. Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. Nature. 2014;510:278-82.

9. Wyce A, Degenhardt Y, Bai Y, Le B, Korenchuk S, Crouthame MC, et al. Inhibition of BET bromodomain proteins as a therapeutic approach in prostate cancer. Oncotarget. 2013;4:2419-29.

10. Stice JP, Wardell SE, Norris JD, Yllanes AP, Alley HM, Haney VO, et al. CDK4/6 Therapeutic Intervention and Viable Alternative to Taxanes in CRPC. Molecular cancer research : MCR. 2017.

11. Kirschner AN, Wang J, van der Meer R, Anderson PD, Franco-Coronel OE, Kushner MH, et al. PIM kinase inhibitor AZD1208 for treatment of MYC-driven prostate cancer. Journal of the National Cancer Institute. 2015;107.

12. Pandit-Taskar N, Veach DR, Fox JJ, Scher HI, Morris MJ, Larson SM. Evaluation of Castration-Resistant Prostate Cancer with Androgen Receptor-Axis Imaging. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2016;57:73S-8S.

13. Bambury RM, Scher HI. Enzalutamide: Development from bench to bedside. Urologic oncology. 2015;33:280-8.

14. O'Donnell KA, Yu D, Zeller KI, Kim JW, Racke F, Thomas-Tikhonenko A, et al. Activation of transferrin receptor 1 by c-Myc enhances cellular proliferation and tumorigenesis. Molecular and cellular biology. 2006;26:2373-86.

15. Huang CH, Lujambio A, Zuber J, Tschaharganeh DF, Doran MG, Evans MJ, et al. CDK9-mediated transcription elongation is required for MYC addiction in hepatocellular carcinoma. Genes & development. 2014;28:1800-14.
16. Holland JP, Evans MJ, Rice SL, Wongvipat J, Sawyers CL, Lewis JS. Annotating MYC status with 89Zr-transferrin imaging. Nature medicine. 2012;18:1586-91.
17. Doran MG, Carnazza KE, Steckler JM, Spratt DE, Truillet C, Wongvipat J, et al. Applying Zr-Transferrin To Study the Pharmacology of Inhibitors to BET Bromodomain Containing Proteins. Molecular pharmaceutics. 2016.
18. Evans MJ. Measuring oncogenic signaling pathways in cancer with PET: an emerging paradigm from studies in castration-resistant prostate cancer. Cancer discovery. 2012;2:985-94.
19. Larson SM, Rasey JS, Allen DR, Nelson NJ, Grunbaum Z, Harp GD, et al. Common pathway for tumor cell uptake of gallium-67 and iron-59 via a transferrin receptor. Journal of the National Cancer Institute. 1980;64:41-53.
20. Behr SC, Aggarwal R, Seo Y, Aparici CM, Chang E, Gao KT, et al. A Feasibility Study Showing [Ga]Citrate PET Detects Prostate Cancer. Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging. 2016.
21. Scher HI, Halabi S, Tannock I, Morris M, Sternberg CN, Carducci MA, et al. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26:1148-59.
22. Sun Y, Niu J, Huang J. Neuroendocrine differentiation in prostate cancer. American journal of translational research. 2009;1:148-62.
23. Truillet C, Cunningham JT, Parker MF, Huynh LT, Conn CS, Ruggero D, et al. Non-invasive measurement of mTORC1 signaling with 89Zr-transferrin. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016.
24. Evans MJ, Holland JP, Rice SL, Doran MG, Cheal SM, Campos C, et al. Imaging tumor burden in the brain with 89Zr-transferrin. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2013;54:90-5.
25. Kumar V, Boddeti DK, Evans SG, Angelides S. (68)Ga-Citrate-PET for diagnostic imaging of infection in rats and for intra-abdominal infection in a patient. Current radiopharmaceuticals. 2012;5:71-5.
26. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. Cancer cell. 2010;18:11-22.
27. Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM, et al. Integrative clinical genomics of advanced prostate cancer. Cell. 2015;161:1215-28.
28. Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrta J, et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nature medicine. 2016;22:298-305.

29. **Hope TA, Aggarwal R, Simko JP, VanBrocklin HF, Ryan CJ. Somatostatin imaging of neuroendocrine-differentiated prostate cancer. Clinical nuclear medicine. 2015;40:540-1.**

Table 1. Distribution of PET-avid metastatic lesions by body site

Organ Site	Number of Lesions on Conventional Imaging	Number of PET-avid lesions on ^{68}Ga-Tf PET (%)	Average SUV_{max} (sd)
Bone	260	166 (63.8%)	5.63 (2.76) Range: 1.8-18.8
Lymph Node	29*	6 (20.7%)	7.04 (2.43) Range: 4.4-10.8
Visceral Organ	37	2 (5.4%)	5.85 (0.07) Range: 5.8-5.9

Table 2. MYC Copy Number Status in Cell Free DNA by Digital PCR Analysis

Samples	# of MYC copies gained	# of MYC copies gained, rounded
PC3 stock (+MYC) control	2.74	3
PC3 cfDNA (+MYC) control	1.58	2
Male germline DNA (WT MYC)	0	0
Patient-001	1.16	1
Patient-003	3.48	3
Patient-004	6.28	6
Patient-005	0.66	1
Patient-006	0.68	1
Patient-007	1.3	1
Patient-008	0.32	0
Patient-010	0.58	1
Patient-011	0.78	1
Patient-012	3.5	4
Patient-013	0.74	1
Patient-014	1.84	2
Patient-015	1.1	1
Patient-016	2.76	3
Patient-017	0.28	0
Patient-018	4.34	4
Patient-019	5.78	6
Patient-021	0.82	1

Figure Legends:

Figure 1. A. A biodistribution study showing the accumulation of ^{68}Ga-citrate in normal mouse tissues and subcutaneous PC3 tumors at 2, 4, and 6 hours post injection. Peak radiotracer uptake was observed in the tumors at 4 hours post injection. **B.** A biodistribution study showing the relative distribution of ^{68}Ga-citrate in normal mouse tissues and subcutaneous 22Rv1 tumors at 4 hours post injection. C. Biodistribution data from mice bearing subcutaneous 22Rv1 tumors showing that the amount of ^{89}Zr-Tf uptake in the tumors is equivalent at 48 hours post injection is equivalent to the amount of ^{68}Ga-citrate uptake in tumors at 4 hours post injection. Importantly, the tumor uptake of ^{89}Zr-Tf exceeded blood pool activity, showing that ~7% ID/g is a value that can represent specific binding.

Figure 2. A. Biodistribution data from nu/nu mice bearing subcutaneous 22Rv1 xenografts shows reduction of tumor uptake of ^{89}Zr-Tf and ^{68}Ga-citrate after treatment with (+)-JQ1 or iBET-151. Mice were treated for 5 days prior to radiotracer administration. Biodistribution studies were conducted 4 hours post injection of ^{68}Ga-citrate, and 48 hours post injection of ^{89}Zr-Tf. Drug treatment continued while ^{89}Zr-Tf equilibrated into peripheral tissues. *$p < 0.01$, ** $p< 0.05$ **B.** A plot showing the equivalent percent reduction in tumor uptake of ^{68}Ga-citrate or ^{89}Zr-Tf due to MYC and TFRC suppression by (+)-JQ1 or iBET-151. **C.** A plot of biodistribution values for ^{68}Ga-citrate in normal muscle (N.M.), an inflamed muscle (I.M.) due to turpentine treatment, and the two prostate cancer tumors shows that radiotracer uptake is higher in tumor.

Figure 3. Distribution of intra-tumoral ^{68}Ga-citrate PET uptake by site of metastasis in the patient cohort. SUVmax = maximum standardized uptake value on PET per lesion

Figure 4. ^{68}Ga-citrate uptake is heterogeneous among lesions detected with conventional imaging in the same patient. A. Multiple regions of uptake on ^{99m}Tc-HDP bone scan (left) including intense uptake in the right femur (blue arrow). While the ^{68}Ga-citrate PET MIP image (right) shows matching uptake in the right femur (blue arrow) it also reveals discordant uptake in the right ischium (orange arrow). **B.** Axial PET, CT, and fused ^{68}Ga-citrate PET/CT through the right ischium show that the CT is normal in region of ^{68}Ga-citrate uptake in the right ischium. CT also showing sclerosis in the region of the abnormal bone scan and ^{68}Ga-citrate uptake (blue arrow).

Figure 5. A maximum intensity projection (MIP) image of Patient-004 demonstrating widepsread uptake within multilevel osseous metastases within the spine, with concurrent cell free DNA evidence of focal, high MYC copy gain

Figure 6. A. Axial PET, CT, and fused ^{68}Ga-citrate PET/Ct showing a ^{68}Ga-citrate avid lesion within the right femur with no CT correlate (blue arrow). **B.** Representative pathology image of the metastasis from the patient's biopsy demonstrateing small cell neuroendocrine prostate cancer.

igure 1